REMARKS

Applicants thank the Examiner for the interview conducted on February 27, 2007, in which the status of the claims was discussed, and for the replacement Office action summary mailed on March 9, 2007. Applicants wish to clarify that claim 3 was cancelled in the reply filed on November 24, 2006; therefore, claims 1-2 and 4-14 are presently pending, and claims 1-2 and 4-8 are under examination. The claims stand rejected under 35 U.S.C. § 101 and § 112, first paragraph. Applicants address the rejections below.

Consideration of Remarks and Entry of Exhibit

Applicants submit herein remarks and an exhibit in order to respond to the present rejections raised by the Office. As consideration of the remarks and entry of the exhibit would serve solely to reduce the issues on appeal and would not necessitate an additional search, applicants respectfully request consideration and entry.

Rejections Under 35 U.S.C. § 101 and § 112, First Paragraph

Claims 1-2 and 4-8 stand rejected under 35 U.S.C. § 101 and § 112, first paragraph, for lack of patentable utility. Applicants respectfully disagree. As a preliminary matter, applicants direct the Office's attention to several passages of M.P.E.P. § 2107 that address the appropriateness of a utility rejection:

> In most cases, an applicant's assertion of utility creates a presumption of utility that will be sufficient to satisfy the utility requirement of 35 U.S.C. 101. (M.P.E.P. § 2107.02.III.A)
>
> If the applicant has asserted that the claimed invention is useful for any particular practical purpose (i.e., it has a "specific and substantial utility") and the assertion would be considered credible by a person of ordinary skill in the art, do not impose a rejection based on lack of utility. (M.P.E.P. § 2107.II.B.1)
>
> An applicant need only provide one credible assertion of specific and substantial utility for each claimed invention to satisfy the utility requirement. (M.P.E.P. § 2107.II.B.1.ii)
>
> Practical considerations require the Office to rely on the inventor's understanding of his or her invention in determining whether and in what regard an invention is believed to be "useful." Because of this, Office personnel should focus on and be receptive to assertions made by the applicant that an invention is "useful" for a particular reason. (M.P.E.P. § 2107.01.I)
>
> Office personnel are reminded that they must treat as true a statement of fact made by an applicant in relation to an asserted utility, unless countervailing evidence can be provided that shows that one of ordinary skill in the art would have a legitimate basis to doubt the credibility of such a statement. (M.P.E.P. § 2107.II)

In view of the above guidelines, and further in view of the assertions and evidence of utility already of record, it is incorrect to maintain the utility rejection. In particular, applicants have established that the compositions falling within the scope of the claims have utility as trypsin family serine proteases, or DNA molecules encoding such proteases. Indeed, the Examiner has acknowledged that "Applicants' arguments provide evidence that their Tespec PRO-2 polypeptide is a trypsin-family protease" (pages 3-4 of the final Office action). Again, note that applicants have made a credible assertion that trypsin family serine protease activity constitutes a specific and substantial utility (see,

e.g., page 4 of the Supplemental Reply filed on January 10, 2007). Furthermore, the compositions of the invention are characterized as belonging to the trypsin family of serine proteases throughout the specification as filed, and even in the title of the application. Based on these facts alone, one of skill in the art would conclude that the claimed compositions have specific and substantial utility.

For clarity, applicants once again direct the Office's attention to Example 10 of the Utility Guidelines, as previously discussed in the Reply filed on November 24, 2006. Example 10 states: "...DNA ligases have a well-established use in the molecular biology art based on this class of protein's ability to ligase DNA." Under Example 10, once it is apparent that an identified protein belongs to a class of proteins having a particular function, and if diseases and/or substrates associated with the proteins that belong to the class are known in the art, such facts are sufficient to establish the specific and substantial utility of the identified protein. It is not necessary to provide additional evidence of specific diseases and/or substrates associated with the identified protein. Furthermore, applicants note that ligases are not all identical in either sequence or function – indeed, a variety of ligases with distinct substrate specificities are known – but the fact that the encoded protein of Example 10 belongs to the class of proteins with DNA ligase activity suffices to establish both specific and substantial utility. No more is required.

Turning to the present case, trypsin-family serine proteases, like DNA ligases, have well-established, specific, and substantial uses in the molecular biology arts, and

skilled artisans would readily recognize the utility of this class of proteins. For example, Nakayama et al. (Drugs of the Future, 22:285-293, 1997; "Exhibit I") teaches that trypsin-like serine proteases, which selectively hydrolyze the C-terminal end of a polypeptide recognizing a basic amino acid residue (Fig. 1), play an important role in the maintenance of the homeostasis of the living body. As illustrated in Fig. 2 of Exhibit I, trypsin-like serine proteases have been reported to play a role in blood coagulation, fibrinolysis, kinin-kallikrein, and complement systems (p. 285, left column, lines 4-10). Exhibit I also describes in detail the activities and functions of the trypsin-like serine proteases (see, e.g., p. 286, Figs. 1 and 2), and further describes specific utilities of trypsin-like serine proteases as targets for drugs. Since specific activities and functions of trypsin-like serine proteases, as well as their utilities as targets for drugs, were known at the time of the present application, a skilled artisan would readily understand the utility of a protein that had been identified as a trypsin-like serine protease.

The Office suggests (page 3) that Example 10 of the Utility Guidelines is inapposite to the present case, because "the protein of SEQ ID NO: 2 does not have high homology to any protein with a demonstrated specific and substantial utility." Applicants disagree. A high degree of sequence homology is not the only way to determine that a protein belongs to a given class. Although sequence homology is indeed one of the parameters that is useful in identifying a protein's function, it is only one available parameter among many. The presence of one or more specific motifs in a sequence can

be another useful method for identifying a function of a protein. The motifs contained in a protein sequence can be critical to a protein's function, and in some cases provide valuable information for identifying that function. In the present case, the evidence submitted with Appendix A of the Supplemental Reply filed on January 10, 2007 states: "[I]f a protein includes both the serine and histidine active site signatures, the probability of it being a trypsin-family serine protease is 100%." Therefore, high homology of the sequence of a protein to any serine protease is not required for the protein to be identified as a trypsin-family serine protease. Accordingly, given the teachings of the scientific literature, it would be unreasonable for the Office to doubt that the protein of SEQ ID NO: 2 having the serine and histidine active site signatures is a trypsin-family serine protease, irrespective of the degree of sequence homology to any other serine protease.

As Example 10 of the Utility Guidelines states: "Based upon applicant's disclosure and the results of the PTO search, there is no reason to doubt the assertion that SEQ ID NO: 2 encodes a DNA ligase." Similarly, in the present case, the specific and substantial utility of the claimed compositions should be acknowledged when no reason to doubt their utility has been presented. If the Office believes that, notwithstanding the evidence of record provided by applicants, there are doubts whether the presently-claimed compositions have specific and substantial utility, applicants respectfully request that the Office provide scientific papers or other evidence supporting these doubts. Applicants submit that the Office's citation of Tucker et al. and Moreno et al. are not material to the

above argument, as these papers discuss prostatin and acrosin, which relate to additional potential utilities of the protein of SEQ ID NO: 2, and provide absolutely no reason to doubt applicants' assertions that the compositions of the presently-claimed invention have trypsin-family serine protease activity, or encode proteins having such activity.

In sum, applicants have established, and the Office has acknowledged, that the presently-claimed compositions constitute trypsin-family serine proteases, or DNA molecules encoding such proteases. These proteases have functions and utilities that are well-known in the art. Accordingly, the compositions of the present invention have specific, substantial, and credible utility, and the rejections under 35 U.S.C. § 101 and § 112, first paragraph, should be withdrawn.

Rejoinder of Withdrawn Claims

As applicants believe that all claims under examination (1-2 and 4-8) are in condition for allowance, applicants respectfully request that withdrawn claims 9 and 11, which are directed to methods of using the products encompassed by claims 1 and 2, be rejoined, in accordance with M.P.E.P. § 821.04.

CONCLUSION

Applicants submit that the claims are in condition for allowance, and such action is respectfully requested. Enclosed is a Petition to extend the period for replying to the Office action for two months, to and including July 13, 2007.

If there are any charges or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Date: 7/13/2007

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EXHIBIT 1

Drugs of the Future 1997, 22(3): 285-293

Review Article

Therapeutic potential of trypsin-like serine protease inhibitors

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CONTENTS

Introduction

Serine proteases possessing a catalytic site consisting of Ser195, His57 and Asp102 have long been known to be involved in the control of biological reactions. Among them, trypsin-like serine proteases, which selectively hydrolyze the C-terminal of the peptide recognizing a basic amino acid residue (Fig. 1), play an important role in the maintenance of the homeostasis of the living body. As illustrated in Figure 2, trypsin-like serine proteases have been reported to control blood coagulation, fibrinolysis, kinin-kallikrein and complement systems (1). The kinin-kallikrein and complement systems have been thought to be closely related to inflammation and immune reactions (2).

Under normal conditions, the body protects itself from the potential damaging effects of proteases with endogenous protease inhibitor (PI). However, if the balance between protease and antiprotease is upset due to a decrease in the levels of PI, the excess protease activity may lead to the development of a disease such as pancreatitis (3), or disseminated intravascular coagulation (4), for example. As shown in Table I, trypsin-like serine proteases are activated in various kinds of diseases (5). As a result, synthetic inhibitors of these excessively released enzymes have been considered useful drugs which can treat or prevent thrombosis, inflammatory diseases, autoimmune diseases and related diseases.

Therapeutic application

Selective synthetic inhibitors such as argatroban **6** have been used clinically for the treatment of thrombosis (6). Many review aticles on the synthetic thrombin inhibitors have been published (7). A selective factor Xa inhibitor, DX-9065a **7** (8), is now in clinical trials. This review focuses on the therapeutic potential of the multiple inhibitors which broadly inhibit trypsin-like serine proteases.

Gabexate mesilate **1** (9), camostat mesilate **2a** (10), nafamostat mesilate **3** (11) and sepimostat mesilate **4** (Fig. 3) have been used clinically. TO-195 **5**, which is considered to be a multiple inhibitor based on its phenol ester-containing structure, is now being studied in a phase III trial as an orally active anticomplement agent (12).

Pancreatitis

Pancreatitis is known as autodigestion of the pancreas caused and exacerbated by the pancreatic proteases (3). As shown in Figure 4, the pancreatic proteases are produced and excreted as their proenzymes (zymogens) in the pancreas and then activated by a small amount of trypsin (13). Thus, these activated pancreatic enzymes digest (hydrolyze) tissue proteins of the organs (pancreas). Pancreatitis has been thought to be a disease caused by the activated enzymes in the pancreas. The mechanism of activation of trypsinogen in the pancreas remains to be clarified. There have been many cases of serious pancreatitis leading to multiple organ failure (14). As a result, the synthetic protease inhibitors, which selectively inhibit trypsin, play an important role in the treatment of pancreatitis, and their usefulness has been confirmed in an animal disease model (15). The intravenous infusion of gabexate has been reported to improve various parameters of pancreatitis and to lower the mortality rate in acute pancreatitis models induced by trypsin and sodium taurocholate in the rat and dog (16). These effects were also reported in caerulein-induced and ethionine-induced acute pancreatitis models (17). Therefore, gabexate has been used clinically for the treatment of acute pancreatitis.

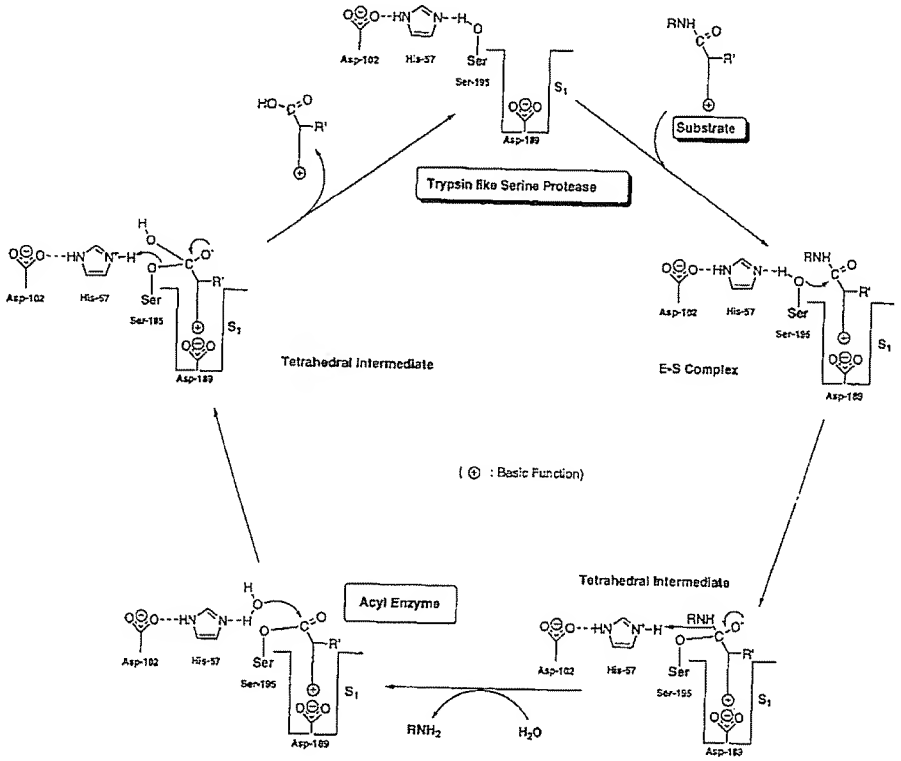


Fig. 1. Cleavage of the peptide by trypsin-like serine protease.

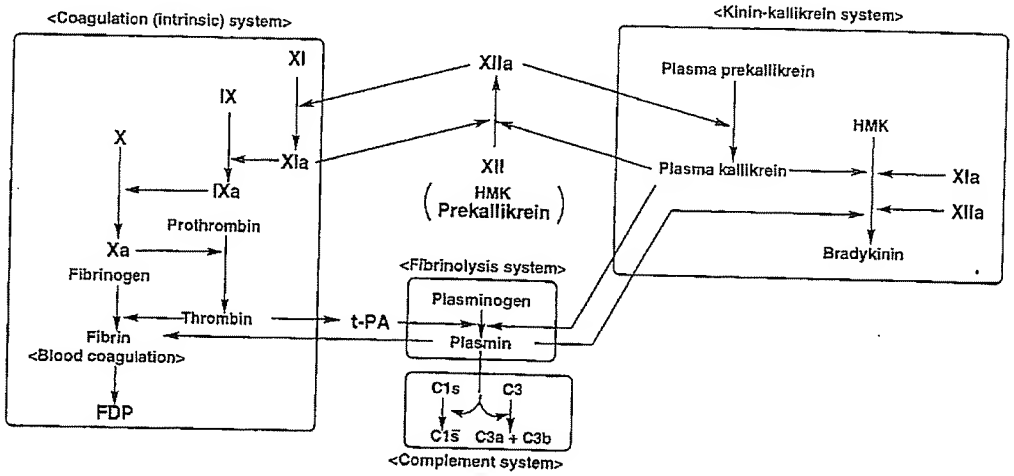


Fig. 2. Interaction of protease cascades related to inflammation in the plasma. The classical, alternative pathway in the complement system and the extrinsic pathway in the coagulation system have been deleted from this figure. tPA: tissue plasminogen activator; FDP: fibrinogen degradation products: HMK: high molecular weight kininogen.

Camostat and sepimostat, which are orally active inhibitors, have been used clinically for the treatment of chronic pancreatitis (18).

Disseminated intravascular coagulation

Disseminated intravascular coagulation (DIC) is a disease caused by serious infectious diseases, malignant

Table I: Trypsin-like serine proteases and their normal functions, related diseases and inhibitors.

Enzyme	Normal function	Related disease	Inhibitor
Trypsin	Digestion	Pancreatitis	Gabexate mesilate, camostat mesilate, nafamostat mesilate
Enterokinase	Activation of trypsin	Pancreatitis	
Factor IXa	Blood coagulation	Vascular clotting, cerebral infarction, coronary infarction	
Factor Xa	Blood coagulation	Vascular clotting, cerebral infarction, coronary infarction	DX-9065a
Factor XIa	Blood coagulation	Vascular clotting, cerebral infarction, coronary infarction	
Factor XIIa	Blood coagulation	Vascular clotting, cerebral infarction, coronary infarction	
Factor VIIa	Blood coagulation	Vascular clotting, cerebral infarction, coronary infarction	
Thrombin	Blood coagulation	Vascular, clotting, cerebral infraction, coronary infarction	Argatroban, others
Plasma kallikrein	Activation of Factor XII, production of kinin		PKSI-527
Plasmin	Fibrinolysis	Tumor invasion	Tranexamic acid
t-PA	Fibrinolysis	Tumor invasion	
u-PA	Fibrinolysis	Tumor invasion	
Factor C1r	Complement activation	Inflammation, rheumatoid arthritis, nephritis	Nafamostat mesilate, sepimostat mesilate, TO-195
Factor C1s	Complemen activation	Inflammation, rheumatoid arthritis, nephritis	Nafamostat mesilate, sepimostat mesilate, TO-195
Factor D	Complement activation	Inflammation, rheumatoid arthritis, nephritis	
Factor B	Complement activation	Inflammation, rheumatoid arthritis, nephritis	
Tryptase (mast cell)	Phagocytosis	Inflammation, emphysema, adult respiratory distress syndrome, rheumatoid arthritis	
Granzyme	Phagocytosis	Inflammation, emphysema, rheumatoid arthritis	

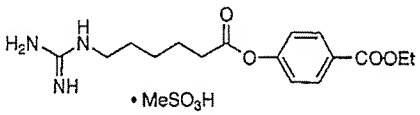
tumors, external wounds and gynecic complications (18). The actual state of such a disease is a marked promotion of the intravascular blood coagulation and the accompanying formation of broad disseminated fibrin in the microvascular blood circulatory system. DIC has also been known to cause organ failure with hemorrhagic tendency induced by the consumption of coagulation factor and blood platelets, and the promotion of second order fibrinolysis. In the process, the balance of thrombin, factor Xa and plasmin is upset. A typical treatment method for DIC is anticoagulation therapy with heparin (19) which promotes the action of antithrombin III (AT-III), a coagulative endogenous protease inhibitor. However, when the concentration of AT-III is less than 50%, the anticoagulative effect of heparin is not enough and lethal hemorrhagic symptoms sometimes occur; this is why the balanced control of blood coagulation and fibrinolysis is needed.

For this purpose, gabexate, possessing balanced inhibitory activity toward thrombin, plasmin and kallikrein besides antitrypsin activity, has been considered useful for the treatment of DIC and has been investigated in various animal models. The inhibitor showed effectiveness not only in experimental DIC models induced by thrombin, tissue thromboplastin or endotoxin, but also on the antishock effect by blocking the formation of microvascular thrombosis in endotoxin shock (20).

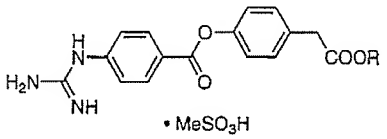
The clinical usefulness of gabexate and nafamostat (with similar profiles in inhibitory spectra and pharmacodynamics) for the therapy and prevention of DIC has been rationalized based on experimental results (9, 11).

Reflux esophagitis after gastrectomy

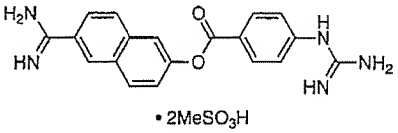
Another therapeutic use of trypsin inhibitors is for the treatment of reflux esophagitis after gastrectomy, which is caused by the reflux of duodenum fluid after gastrectomy. Salo *et al.* reported that the causative factor in this disease



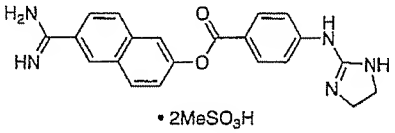
1 (Gabexate mesilate)



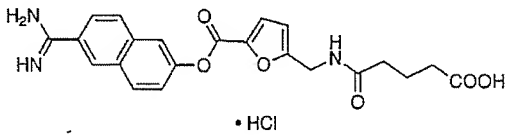
2a R = CH_2CONMe_2 (Camostat mesilate)
2b R = H (FOY-251)



3 (Nafamostat mesilate)



4 (Sepimostat mesilate)



5 (TO-195)

Fig. 3. Synthetic trypsin-like serine protease inhibitors.

is the synergism of taurocholic acid and trypsin contained in the fluid refluxed directly from the duodenum (21). The orally active trypsin inhibitors camostat and sepimostat were demonstrated to be effective in the prevention and treatment of reflux esophagitis after gastrectomy in experimental models using rats and dogs (22).

Miscellaneous

1) Nephritis

Most of the reported cases of intraglomerular nephritis have been thought to be induced by disorders of the immune system and to deteriorate through the mutual close participation of blood coagulation-fibrinolysis, kinin and complement systems. As such, clinical therapy by the suppression of these damaging mediators and platelet aggregation has been tested. Camostat has been reported to reduce the urinary protein of patients with diabetic nephritis or nephrosis syndrome (23). TO-195, possessing anticomplement activity, is now under evaluation for the treatment of plasma-induced Masugi disease in rabbits and chronic serum-induced nephritis in rats. Oral administration of the compound improved the urinary parameters of nephritis (12).

2) Inflammatory bowel disease

Ulcerative colitis and Crohn's disease, typical examples of inflammatory bowel disease, which is induced mainly in the intestine of young adults, have been considered to be autoimmune diseases. Although an effective therapeutic method for the treatment of these diseases has not yet been established, camostat was effective against a sodium dextran sulfate-induced ulcerative colitis model in the rat (24). As a result, an overactivation of trypsin-like serine proteases in these diseases was strongly suggested. The clinical effectiveness of camostat against ulcerative colitis was also reported (25).

3) Ischemic heart disease

Nafamostat, possessing potent anticomplement activity which is structurally derived from amidinonaphthyl benzoate, has been reported to suppress tissue injury caused by the activation of complement in an ischemia-reperfusion model of isolated rabbit heart (26). Based on these results, nafamostat, sepimostat and TO-195 are expected to be effective against ischemic heart disease.

ONO-3403 and its active metabolite

ONO-3403 **8a** and its active metabolite **8b** (Fig. 5) are newly developed orally active trypsin-like serine protease inhibitors (27,28) possessing a broader inhibitory spectrum than those of the known inhibitors. Their potent LTB_4 antagonist activity (29) and mite protease inhibitory activity (30) have also been reported, and therefore their greater potential therapeutic use is expected.

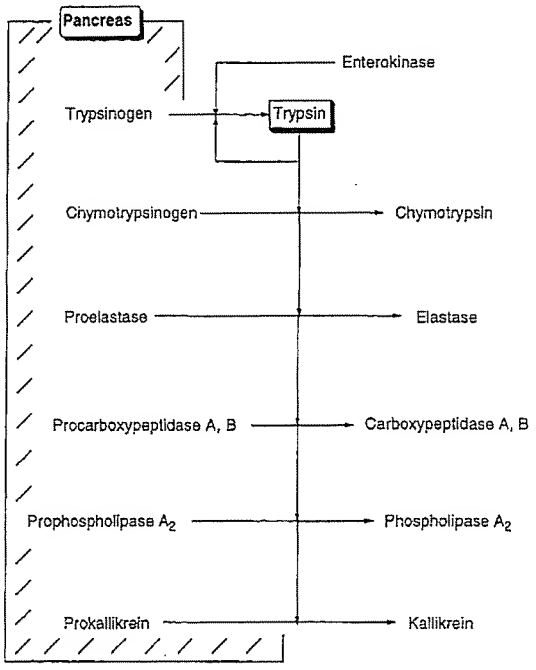


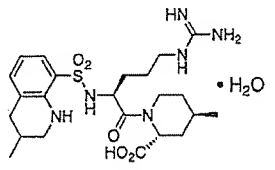
Fig. 4. Activation mechanism of proenzymes in pancreas.

Pharmacodynamics and biological profiles

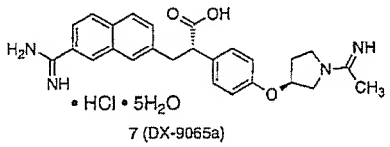
The trypsin-like serine protease inhibitors described above have been shown to be therapeutically useful drugs for certain kinds of diseases. Their clinical utility for a broader spectrum of diseases is limited mainly because of their pharmacodynamics such as duration of action, tissue delivery and oral activity. Maintaining the necessary blood level of these inhibitors for the treatment of other diseases is usually difficult because of their poor oral absorption and metabolic instability. As shown in Figure 6, the new inhibitors ONO-3403 **8a** and its metabolite **8b** showed much better oral activity and duration of action than camostat in rats. In dogs, the metabolite showed good oral absorption (Fig. 7), whereas parent compound did not. After an extensive investigation of their pharmacodynamics, ONO-3403 was shown to be orally absorbed mainly after its enzymatic hydrolysis to the metabolite in the intestine (Table II). Only species such as rats, pigs, monkeys and humans possessing a metabolic enzyme which converts ONO-3403 to its metabolite in their intestine can absorb ONO-3403 as its active metabolite (27).

Both ONO-3403 and its metabolite showed broader inhibitory spectra and more potent inhibitory activity than camostat and FOY-251 **2b**, as shown in Table III. In fact, camostat showed dose-response efficacy in a trypsin-sodium taurocholate-induced pancreatitis model in rats (31) (Fig. 8).

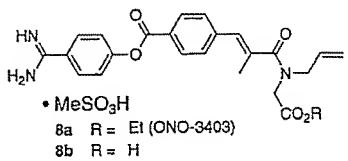
ONO-3403 and its metabolite were expected to be effective on mite-induced allergy based on their potent inhibitory activity toward *Dermatophagodes fainane* (Df) protease (30) (Table III). Df protease from the house dust mite is closely associated with mite-induced allergy, having a similar substrate specificity to blood coagulation factor XIIa and catalyzing the activation of kinin-kallikrein system in human plasma. With the goal of the prevention of kinin formation in plasma by Df protease, the inhibition of Df protease with synthetic inhibitors was tested *in vivo* and *in vitro*. Of the inhibitors, including amidine and guanidine derivatives, ONO-3403 was the most effective at inhibiting Df protease ($K_i = 9 \times 10^{-9}$ M) and preventing kinin release from Df protease in human plasma. The enhancement of vascular permeability in guinea pigs caused by kinin release was stoichiometrically suppressed by the inhibitor (30). The inhibitory activity of ONO-3403 and its metabolite toward other trypsin-like serine proteases and their therapeutic



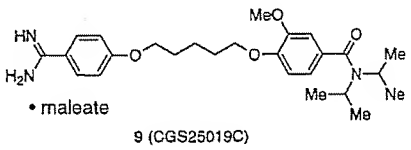
6 (Argatroban)



7 (DX-9065a)



8a R = Et (ONO-3403)
8b R = H



9 (CGS25019C)

8a R = Et (ONO-3403)
8b R = H

Fig. 5. Synthetic trypsin-like serine protease inhibitors and LTB_4 receptor antagonist.

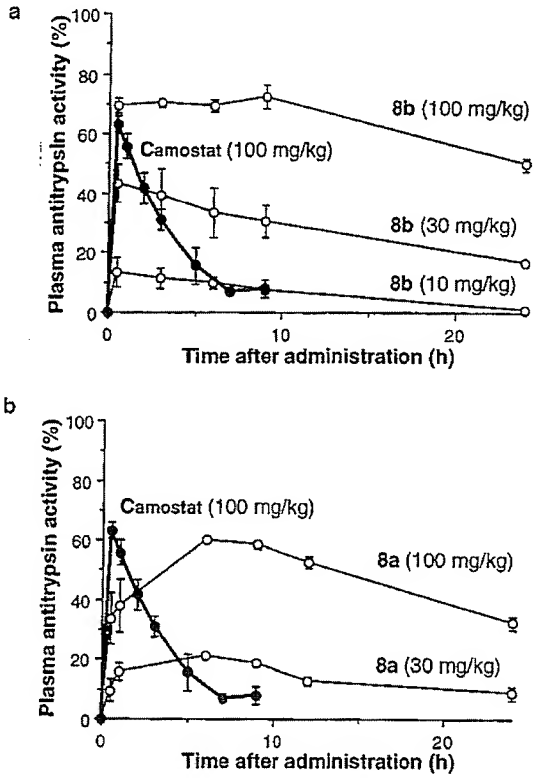


Fig. 6. (a) Plasma antitrypsin activity in fasted rats (n = 3) after oral administration of **8b**. After administration, blood was taken from the jugular vein at regular intervals. To the plasma (0.1 ml) was added bovine pancreatic trypsin (2 μM/ml, 0.1 ml) followed by the substrate Boc-Phe-Ser-Arg-AMC (0.1 mM, 0.8 ml). The reaction mixture was incubated at 37 °C for 15 min and quenched by addition of 30% acetic acid (1 ml) to obtain samples for analysis. Fluorescence of the released 7-amino-4-methylcoumarin (AMC) in the samples was measured (excitation: 380 nm, emission: 460 nm) using a Hitachi 850 fluorescence spectrometer. Plasma antitrypsin activity (%) was calculated according to the following equation:

$$\text{Inhibition (\%)} = \frac{\text{Pre (F)} - \text{Test (F)} \times 100}{\text{Pre(F)}}$$

where Pre(F) is fluorescence of the control group (no drug administered) and Test (F) is fluorescence of the tested group. (b) Plasma antitrypsin activity in rats (n = 3) after oral administration of **8a**. Plasma antitrypsin activity of **8a** was evaluated as described in the legend of Fig. 6a.

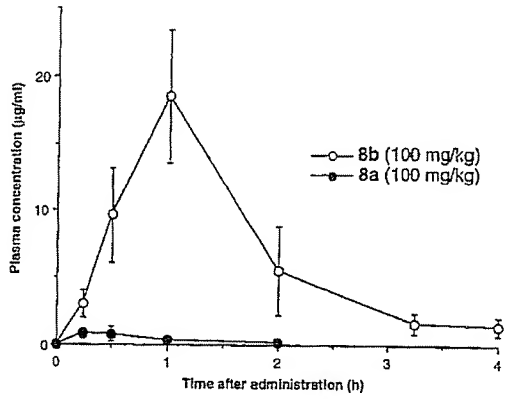


Fig. 7. Plasma concentration after oral administration of **8a** and **8b** to beagle dogs. Plasma concentration in fasted beagle dogs was determined by HPLC analysis. Values are shown as means ± SD (n = 3). For **8b**, plasma concentration was shown as **8a** + **8b** (eq. μg/ml).

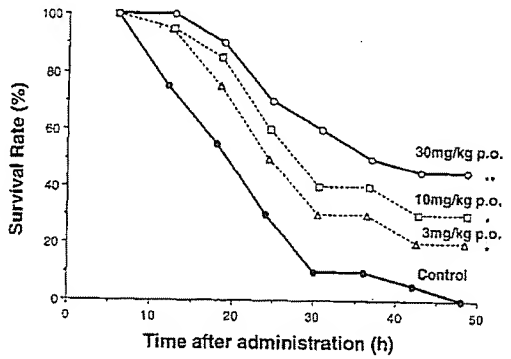


Fig. 8. Effect of **8a** (n = 10) on survival rate of rats with pancreatitis induced by trypsin-sodium taurocholate. **8a** was given orally to the rats 30 min before induction of pancreatitis. $^{*}p < 0.05$; $^{**}p < 0.01$ (log-rank method).

utility based on the newly found inhibitory activity are now under investigation in our laboratory.

Leukotriene B_4 (LTB_4) receptor antagonist activity of ONO-3403 and its metabolite was discovered based on their structural similarity to CGS-25019C (**9**) (29). LTB_4 has been reported to be widely involved in inflammatory, allergic and immune diseases (29). ONO-3403 and its metabolite inhibited [^{3}H]-LTB_4 binding to the LTB_4 receptor in human neutrophils (PMN) with K_i values of 33.0 and 105.0 nM, respectively, and selectively inhibited LTB_4-induced human PMN intracellular calcium mobilization (IC_{50} = 106.6 ± 33.8 and 115.0 ± 21.0 nM, respectively) and degranulation (IC_{50} = 16.4 ± 2.5 and 103.9 ± 234.1 nM, respectively) (29). Based on these additional biological activities, other potential therapeutic applications of ONO-3403 and its metabolite are now under investigation in our laboratory.

Proposed mechanisms of action

Trypsin and the newly designed and orally active synthetic trypsin inhibitor **8b** were cocrystallized. Since the refined model of the **8b**-trypsin complex provides the structural basis of X-ray results, it is proposed that the potent

*Table II: Metabolism of **8a** and **8b** in the tissue homogenates of rat and dog.*

Compound	Animal	Small intestine homogenate $t_{1/2}$ (min)	Plasma $t_{1/2}$ (min)	Liver homogenate $t_{1/2}$ (min)
8a	Rat	11[a]	< 1[b]	< 1[b]
8b	Rat	> 60	> 60	> 60
8a	Dog	> 60	25[c]	< 1[b]
8b	Dog	> 60	15[d]	> 60

[a]**8a** → **8b** + amidinophenol + R_1. [b]**8a** → **8b**. [c]**8a** → amidinophenol + R_1. [d]**8b** → amidinophenol + R_2.

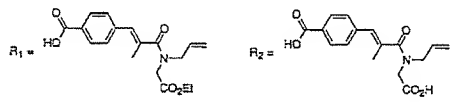


*Table III: Inhibitory spectrum of the synthetic inhibitors **1**, **2a-b**, **3**, **4**, **8a-b** and **5** (27, 32).*

	IC_{50} (K_i) μM									
Compound	Trypsin	Thrombin	Plasmin	Plasma kallikrein	Pancreatic kallikrein	Chymotrypsin	C1r	C1s	Df protease	LTB_4 receptor binding
1	(0.22)	(0.97)	(1.60)	(0.20)	(120)	(67)	NT[a]	NT[a]	NT[a]	NT[a]
2a	0.051	31.6	2.62	1.48	34.1	> 100	NT[a]	NT[a]	NT[a]	NT[a]
2b	0.055	107	12.5	2.32	> 100	> 100	NT[a]	NT[a]	NT[a]	NT[a]
3	0.027	0.5	0.14	0.0021	12	150	0.18	0.030	NT[a]	NT[a]
	(0.24)	(1.2)	(0.26)	(0.057)	(1.2)	(4.0)				
4	2.7	6.4	1.9	0.26	3.6	280	23	0.20	NT[a]	NT[a]
	(0.097)	(2.5)	(0.57)	(0.61)	(0.029)		(6.4)	(0.18)		
8a	0.011	3.66	0.873	0.19	15.7	> 100	NT[a]	NT[a]	(0.009)	0.063
8b	0.0041	35	1.8	0.097	> 100	> 100	NT[a]	NT[a]	(0.01)	0.201
5	0.43	0.035	0.43	NT[a]	0.47	NT[a]	0.049	0.25	NT[a]	NT[a]

The following compounds were used as the synthetic substrates of each protease: Boc-Phe-Ser-Arg-AMC for trypsin; H-D-Phe-Pip-Arg-pNA for thrombin; H-D-Val-Leu-Arg-pNA for pancreatic kallikrein; H-D-Pro-Phe-Arg-pNA for plasma kallikrein; H-D-Val-Leu-Lys-pNA for plasmin; Suc-Ala-Ala-Pro-Phe-pNA for chymotrypsin; Ac-Arg-Me for C1r; Ac-Gly-Lys-Me for C1s (AMC = 7-amino-4-methylcoumarin, pNA = *p*-nitroaniline). [a]Not tested.

inhibitory activity of **8b** is due mainly to the formation of an acylated trypsin through an inverse substrate mechanism and its low rate of deacylation (28).

A comparison of the crystal structure of the **8b**-trypsin complex with that of guanidinobenzoic acid (GB)-trypsin complex, which was formed by the cocrystallization of trypsin and *p*-nitrophenyl guanidinobenzoate, indicated that the hydrogen bond network at the catalytic triad (Fig. 9), which is usually observed in the substrate-trypsin complex, is not present in the complex of trypsin with the inverse substrate-type inhibitor. Instead, the three residues of His57, Asp102 and Ser195 are linked to one another by hydrogen bonds

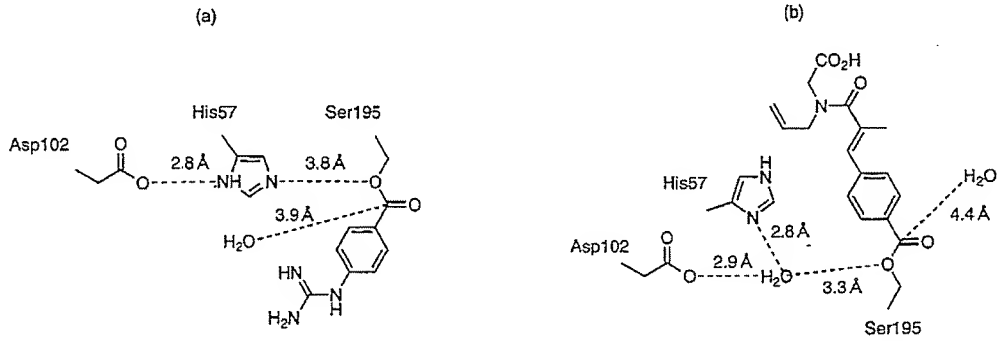


Fig. 9. Schematic comparison of the catalytic triad moieties in trypsin acylated with *p*-nitrophenyl *p*-guanidinobenzoate (a) and **8b** (b).

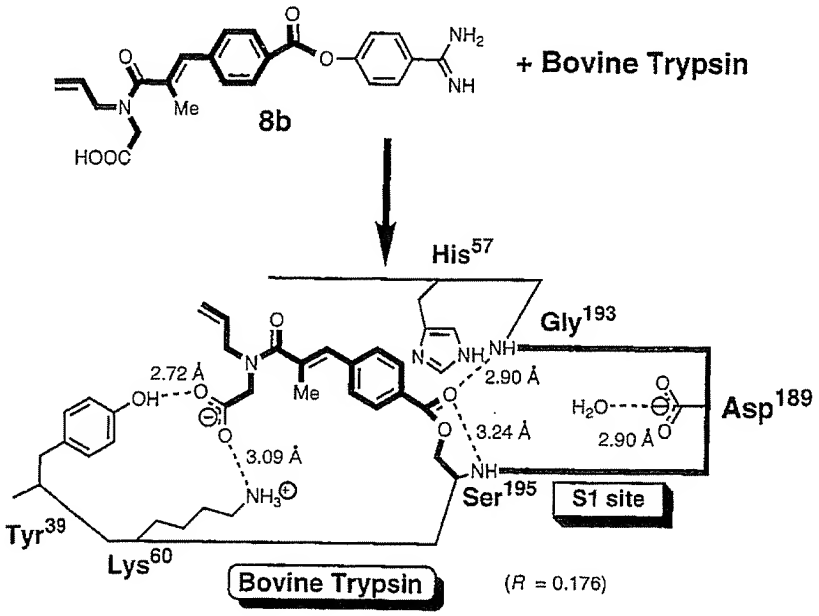


Fig. 10. Schematic hydrogen bond network formed in the **8b**-trypsin complex.

via one water molecule. This leads us to speculate that the rate of hydrolysis of the **8b**-trypsin complex is lower than that of the GB-trypsin complex.

Possible hydrogen bonds of **8b** in the trypsin-binding pocket are schematically shown in Figure 10. The carbonyl oxygen atom of the acylated **8b** is located in the oxyanion hole (28) and forms hydrogen bonds with the NH groups of Gly193 and Ser195. The acyl moiety of **8b** is in Van der Waals contact with the sulfur atom of the S-S bond formed between Cys42 and Cys58. Two carboxylic oxygen atoms of the *N*-allylglycine of **8b** form hydrogen bonds with the hydroxyl group of Tyr39 and the Nε amino group of Lys60.

In conclusion, the reaction mechanism of the inverse substrate-type inhibitor **8b**, which exhibits potent and long-lasting activity, might involve the following four sequential steps: 1) recognition of the basic group by trypsin, 2) formation of a Michaelis complex, 3) formation of an acylated trypsin, and 4) slow deacylation, accompanied by formation of a tetrahedral intermediate by the addition of a water molecule to the ester carbonyl carbon atom of **8b** fragment linked to Ser195.

Conclusions

Many biological roles of the trypsin-like serine proteases remain to be clarified. In this article we have reviewed the very limited therapeutic use of their inhibitors. The therapeutic application of these inhibitors may be extended by identifying as yet unknown profiles in their inhibitory spectra and/or pharmacodynamics, such as in ONO-3403 and its metabolite (**8b**). Inhibitors with both specific and broad inhibitory spectra must be developed to disclose the therapeutic potential of these drugs.

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